

TECHNICAL BULLETIN

CellTiter-Blue[®] Cell Viability Assay

Instructions for Use of Products
G8080, G8081 and G8082



CellTiter-Blue[®] Cell Viability Assay

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1. Description

The CellTiter-Blue[®] Cell Viability Assay provides a homogeneous, fluorometric method for estimating the number of viable cells present in multiwell plates. It uses the indicator dye resazurin to measure the metabolic capacity of cells—an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent (Figure 1). Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal.

The CellTiter-Blue[®] Reagent is a buffered solution containing highly purified resazurin. The ingredients have been optimized for use as a cell viability assay. The spectral properties of CellTiter-Blue[®] Reagent change upon reduction of resazurin to resorufin (Figure 2). Resazurin is dark blue in color and has little intrinsic fluorescence until it is reduced to resorufin, which is pink and highly fluorescent (579_{Ex}/584_{Em}).

The visible light absorbance properties of CellTiter-Blue® Reagent undergo a “blue shift” upon reduction of resazurin to resorufin. The absorbance maximum of resazurin is 605nm and that of resorufin is 573nm. Either fluorescence or absorbance may be used to record results; however, fluorescence is the preferred method because it is more sensitive and involves fewer data calculations.

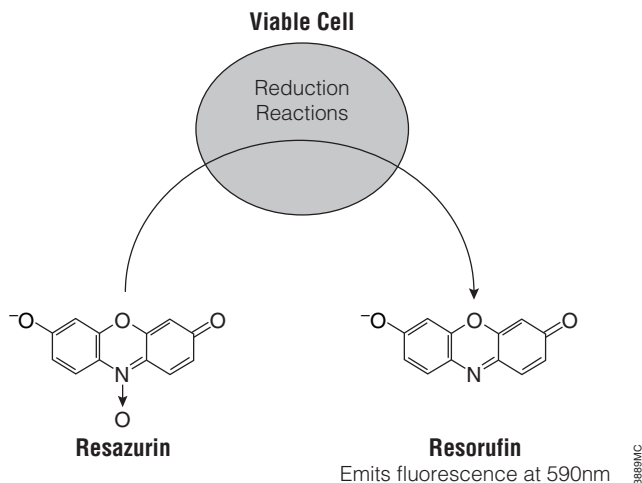


Figure 1. Conversion of resazurin to resorufin by metabolically active cells results in the generation of a fluorescent product. The fluorescence produced is proportional to the number of viable cells.

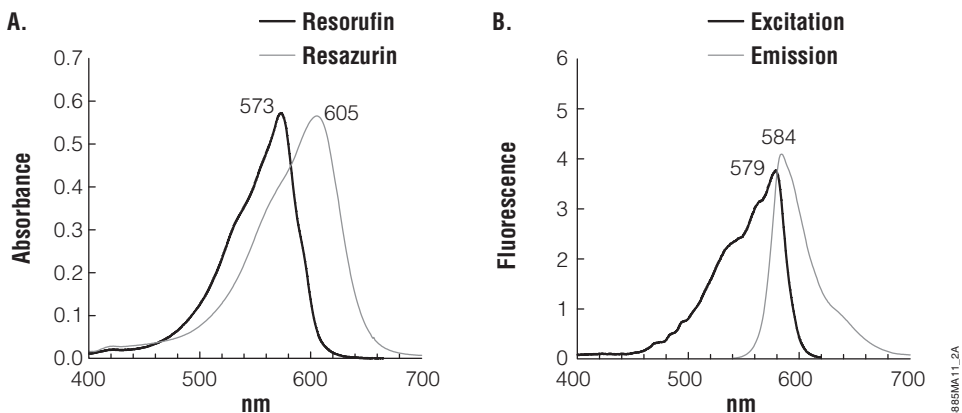


Figure 2. Spectral properties of resazurin and resorufin in RPMI + 10% fetal bovine serum. Panel A. Absorbance spectra for resazurin and resorufin. **Panel B.** Fluorescence excitation and emission spectra for resorufin.

A flow diagram summarizing the CellTiter-Blue® Assay protocol is shown in Figure 3. The homogeneous assay procedure involves addition of a single reagent (CellTiter-Blue® Reagent) directly to cells cultured in serum-

supplemented medium. After an incubation step, data are recorded using either a fluorometer or spectrophotometer. Cell washing, removal of medium and multiple pipetting steps are not required, making the assay ideal for adaptation to automated high-throughput screening for cell viability and cytotoxicity.

Under most experimental conditions, the fluorescent signal from the CellTiter-Blue® Reagent is proportional to the number of viable cells. There is a linear relationship between cell number and fluorescence. The linear range and lower limit of detection are dependent on the cell type and the ability to reduce resazurin (Figure 4).

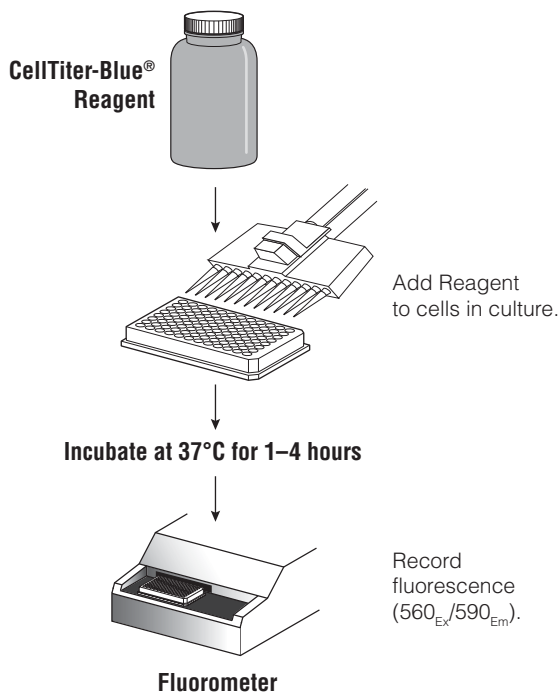


Figure 3. The CellTiter-Blue® Cell Viability Assay protocol. Multiwell plates (96- or 384-well) compatible with fluorescent plate readers are prepared with cells and the compounds to be tested using standard methods. CellTiter-Blue® Reagent is added directly to each well, the plates are incubated at 37°C to allow cells to convert resazurin to resorufin, and the fluorescent signal is measured.

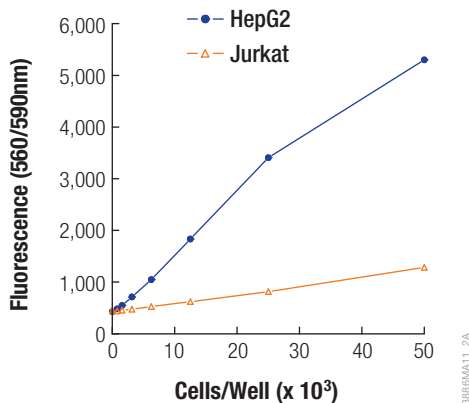


Figure 4. Relative ability of different cell types to reduce resazurin. Serial twofold dilutions of Jurkat or HepG2 cells were prepared at 100µl/well in a 96-well plate and cultured for 1.5 hours at 37°C. CellTiter-Blue® Reagent (20µl/well) was added and cells were incubated for 1 hour before recording fluorescence (560(20)_{Ex}/590(10)_{Em}) using a Labsystems Fluoroskan Ascent plate reader.

The range of linear responsiveness also is dependent on the ratio of CellTiter-Blue® Reagent to culture medium and the incubation time. Figure 5, Panel A, shows that, when using the recommended dilution of CellTiter-Blue® Reagent, at 4 hours of incubation there was a linear response ($r^2 = 0.99$) from 0–50,000 Jurkat cells/well in the 96-well format. When the incubation period was extended to 22 hours, the signal-to-background ratio improved for lower cell numbers, leading to increased assay sensitivity, but there was no longer a linear relationship with fluorescence at high cell numbers.

The data in Figure 5, Panel B, were used to calculate Z' factor values as an indication of the suitability of the CellTiter-Blue® Assay for high-throughput screening (1). The Z' factor value calculated using data from 4 hours of incubation for 780 Jurkat cells/well was 0.68. Higher concentrations of cells (1,562–50,000 cells/well) had Z' factor values between 0.74 and 0.98. The Z' factor values improved with longer incubation times for lower cell concentrations. At 22 hours of incubation, the Z' factor value for 195 cells/well was 0.64. Higher concentrations of cells had Z' factor values between 0.7 and 0.99 after 22 hours of incubation with CellTiter-Blue® Reagent.

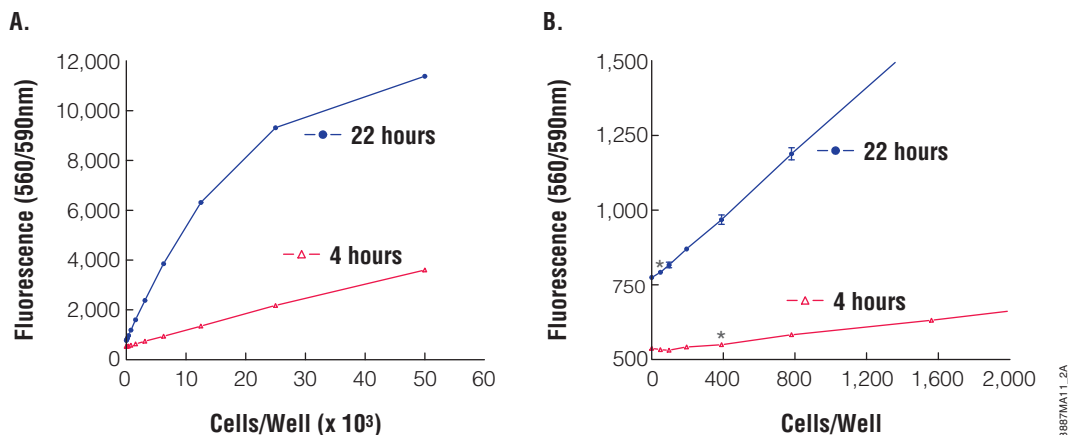


Figure 5. Effect of CellTiter-Blue[®] Assay incubation time on signal formation. Serial twofold dilutions of Jurkat cells from 0–50,000 were incubated with CellTiter-Blue[®] Reagent for 4 hours and 22 hours. **Panel A.** The 4-hour incubation period shows a linear correlation ($r^2 = 0.99$) between fluorescence and cell number. The lower limit of detection was 390 cells/well. For the 22-hour incubation period, there is a gain in assay sensitivity with a lower limit of detection of 49 cells/well but a loss of linearity above 12,500 cells/well. **Panel B.** Detail for 0–2,000 cells per well. For the 4-hour incubation, the signal from 390 cells (*) was greater than that from zero cells + 3 standard deviations. For the 22-hour incubation, the signal from 49 cells (*) was greater than that from zero cells + 3 standard deviations.

Advantages of the CellTiter-Blue[®] Assay

- **Saves You Time:** The homogeneous add-incubate-measure format reduces the number of handling steps.
- **Allows Your Choice of Assay Format and Method of Detection:** Can be used with 96- or 384-well formats and data can be recorded using fluorescence (preferred) or absorbance.
- **Allows Performance of More than One Assay on the Same Sample:** Can be multiplexed with other assay methods such as the Apo-ONE[®] Homogeneous Caspase-3/7 Assay (Cat.# G7790) for detecting apoptosis (see Section 3.F).
- **Convenient—Whatever Your Throughput Needs:** The reagent has been designed to provide sufficient volumes for accurate pipetting into 96- or 384-multiwell formats. Convenient product sizes available for high-throughput screening.
- **Safe:** The reagent is generally nontoxic to cells, allowing extended incubation periods in some situations (see Section 3.A). Requires no scintillation cocktail, radioactive waste disposal (unlike [³H]-thymidine incorporation assays) or use of hazardous solvents (required for MTT-based assays).



2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
CellTiter-Blue® Cell Viability Assay	20ml	G8080

Each system contains sufficient reagents to perform 1,000 assays in a 96-well format or 4,000 assays in a 384-well format when the recommended volumes are used. Includes:

- 1 × 20ml CellTiter-Blue® Reagent

PRODUCT	SIZE	CAT. #
CellTiter-Blue® Cell Viability Assay	100ml	G8081

Each system contains sufficient reagents to perform 5,000 assays in a 96-well format or 20,000 assays in a 384-well format when the recommended volumes are used. Includes:

- 1 × 100ml CellTiter-Blue® Reagent

PRODUCT	SIZE	CAT. #
CellTiter-Blue® Cell Viability Assay	10 × 100ml	G8082

Each system contains sufficient reagents to perform 50,000 assays in a 96-well format or 200,000 assays in a 384-well format when the recommended volumes are used. Includes:

- 10 × 100ml CellTiter-Blue® Reagent

Storage Conditions: Store the CellTiter-Blue® Reagent frozen at –20°C protected from light. Thaw CellTiter-Blue® Reagent completely and mix thoroughly before use. For frequent use, the product may be stored tightly capped at 4°C or at ambient temperature (22–25°C) for 6–8 weeks. Protect from light. The product is stable for at least 10 freeze-thaw cycles.

3. General Considerations

3.A. Incubation Time

The ability of different cell types to reduce resazurin to resorufin varies depending on the metabolic capacity of the cell line and the length of incubation with the CellTiter-Blue® Reagent. For most applications a 1- to 4-hour incubation period is adequate. For optimization of assay performance for screening assays, the number of cells/well and the length of the incubation period should be empirically determined.

The CellTiter-Blue® Reagent is designed for use as an endpoint assay rather than a kinetic method of monitoring cell growth. The reagent should be added near the end of the period of exposure to the compound being tested. Exposure of cells to resazurin-containing solutions for several days has been reported as a method to monitor cell proliferation. However, extreme precaution should be taken performing such experiments and interpreting those data. While there have been reports in the literature suggesting that solutions containing resazurin are not toxic to cells (2), other reports clearly show that resazurin affects cell viability, depending on the concentration and length

of exposure (3,4).

If cells are exposed to a compound that requires 24 hours to induce cytotoxicity, and are simultaneously exposed to resazurin, they may retain the ability to reduce resazurin and produce a fluorescent signal during the first several hours of exposure to the toxin. Artifacts have been noted for cases where exposure to such compounds for 24 hours resulted in generation of fluorescence, but light microscopy showed 100% cell death (5).

As shown in Figure 5 (and reference 6), longer incubation periods expand the detectable range of the assay by increasing sensitivity. Assays using low cell numbers will produce little fluorescent signal during a two-hour incubation period with resazurin; however, extending the incubation period to several hours may result in an improved signal-to-background ratio and an improvement in the lower limit of detection.

When high numbers of cells are incubated with solutions containing resazurin for extended periods of time, a secondary reduction reaction may occur in which the fluorescent resorufin is further reduced to the colorless, nonfluorescent hydro-resorufin (5,7). This secondary reduction phenomenon may be dependent on the cell type used.

3.B. Volume of CellTiter-Blue® Reagent Used

The recommended quantity of CellTiter-Blue® Reagent to use is 20µl of reagent to each 100µl of medium in a 96-well format, and 5µl of Reagent to each 25µl of culture medium in a 384-well format. This ratio of CellTiter-Blue® Reagent:cell culture volume resulted in a greater fluorescent signal and a reduced fluorescent background compared to other commercially available resazurin-based assays using the Labsystems Fluoroskan Ascent plate reader with a 560(20)_{Ex}/590(10)_{Em} filter set. The ratio of CellTiter-Blue® Reagent:cell culture volume should be adjusted for optimal performance depending on the cell type, incubation time and linear range desired.

3.C. Site of Resazurin Reduction

Microscopy has shown that resazurin is reduced to resorufin inside living cells (5). Resazurin can penetrate cells, where it becomes reduced to the fluorescent resorufin, probably as a result of the action of several different redox enzymes. The fluorescent resorufin dye can diffuse out of cells and back into the surrounding medium. Culture medium harvested from rapidly growing cells does not reduce resazurin (5). An analysis of the ability of various hepatic subcellular fractions suggests that resazurin can be reduced by mitochondrial, cytosolic and microsomal enzymes (8).

3.D. Optical Properties of Resazurin and Resorufin

Absorbance and Fluorescence

Both the light absorbance and fluorescence properties of the CellTiter-Blue® Reagent are changed by cellular reduction of resazurin to resorufin, thus either absorbance or fluorescence measurements can be used to monitor results.

Figure 2 shows the absorption and fluorescence emission spectra of resazurin and resorufin in serum-supplemented culture medium. The absorption maximum for resazurin is 605nm, and the absorption maximum for resorufin is 573nm. If absorbance measurements are used to record data, we recommend taking readings at

3.D. Optical Properties of Resazurin and Resorufin (continued)

570nm and using 600nm as a reference wavelength. Values can be compared to blank wells containing CellTiter-Blue® Reagent without cells. Note: The absorption maxima for resazurin and resorufin are relatively broad, and alternative wavelengths may prove useful if your instrument does not contain 570nm and 600nm filters.

We recommend fluorescence as the preferred method for recording data because it is much more sensitive than using absorbance measurements and requires fewer calculations to account for the overlapping absorbance spectra of resazurin and resorufin. Options for fluorescence filter sets include 530–570nm for excitation and 580–620nm for fluorescence emission.

3.E. Background Fluorescence and Light Sensitivity of Resazurin

The resazurin dye (blue) in the CellTiter-Blue® Reagent and the resorufin product (pink) are light-sensitive. Prolonged exposure of the CellTiter-Blue® Reagent to light will result in increased background fluorescence in the assay and decreased sensitivity. Background fluorescence can be corrected by including control wells on each plate to measure the fluorescence from serum-supplemented culture medium in the absence of cells. There may be an increase in background fluorescence in wells without cells after several hours incubation.

3.F. Multiplexing with Other Assays

Because CellTiter-Blue® Reagent is relatively non-destructive to cells during short-term exposure, it is possible to use the same culture wells to do more than one type of assay. An example showing the measurement of cell viability using the CellTiter-Blue® Reagent and the measurement of apoptosis using the Apo-ONE® Homogeneous Caspase-3/7 Assay is shown in Figure 6.

3.G. Stopping the Reaction

The fluorescence generated in the CellTiter-Blue® Assay can be stopped and stabilized by the addition of 3% SDS. We recommend adding 50µl per 100µl of original culture volume. The plate can then be stored at ambient temperature for up to 24 hours before recording data, provided that the contents are protected from light and covered to prevent evaporation.

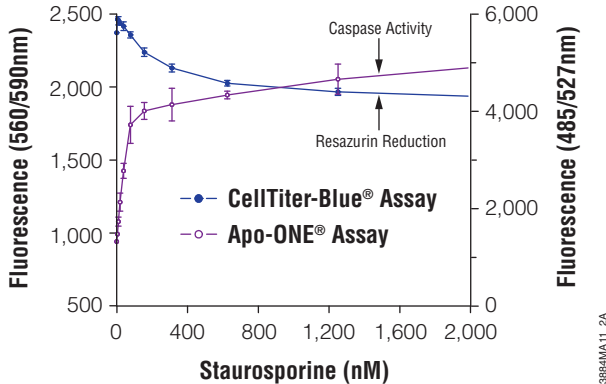


Figure 6. Multiplexing two assays in the same well. Jurkat cells were treated with various concentrations of staurosporine. CellTiter-Blue® Reagent was added to each well immediately after drug addition, and the cells were incubated for 5 hours prior to recording fluorescence ($560_{\text{Ex}}/590_{\text{Em}}$). Then caspase activity was measured in the same wells by adding 120 μ l of the Apo-ONE® Homogeneous Caspase 3/7 Assay Reagent (9). Cells were incubated for an additional hour at ambient temperature prior to recording fluorescence ($485_{\text{Ex}}/527_{\text{Em}}$).

4. Protocol

Materials to Be Supplied by the User

- 96- or 384-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reservoirs to hold CellTiter-Blue® Reagent
- fluorescence reader with excitation 530–570nm and emission 580–620nm filter pair
- (optional) absorbance reader with 570nm and 600nm filters

4.A. Reagent Preparation

Thaw CellTiter-Blue® Reagent and bring to ambient temperature. A 37°C water bath may be used to thaw the reagent. Protect the CellTiter-Blue® Reagent from direct light.

4.B. Recommended Controls

No-Cell Control: Set up triplicate wells without cells to serve as the negative control to determine background fluorescence that may be present.

Untreated Cells Control: Set up triplicate wells with untreated cells to serve as a vehicle control. Add the same solvent used to deliver the test compounds to the vehicle control wells.

Optional Test Compound Control: Set up triplicate wells without cells containing the vehicle and test compound to test for possible interference with the CellTiter-Blue® Reagent chemistry.

Positive Control for Cytotoxicity: Set up triplicate wells containing cells treated with a compound known to be toxic to the cells used in your model system.

4.C. Cytotoxicity Assay Protocol

1. Set up 96-well assay plates containing cells in culture medium.
Note: For 384-well plates, the recommended culture volume is 25µl per well. Add 5µl per well of CellTiter-Blue® Reagent.
2. Add test compounds and vehicle controls to appropriate wells so the final volume is 100µl in each well.
3. Culture cells for the desired test exposure period.
4. Remove assay plates from 37°C incubator and add 20µl/well of CellTiter-Blue® Reagent.
5. Shake for 10 seconds.
6. Incubate using standard cell culture conditions for 1–4 hours.
Note: Extended incubation periods may be used for some applications.
7. Shake plate for 10 seconds and record fluorescence at 560/590nm.

4.D. Calculation of Results

Fluorescence Data

1. **Optional:** Subtract the average of fluorescence values of the culture medium background from all fluorescence values of experimental wells.
Note: Fluorescence can be stopped and stabilized as detailed in Section 3.G.
2. Plot fluorescence versus concentration of test compound.

Absorbance Data

1. Subtract the average of absorbance 600nm values of the culture medium background from all absorbance 570nm values of experimental wells.
2. Plot 570–600nm absorbance versus concentration of test compound.

5. Comparison with Other Assay Methods

There is an excellent correlation among data from the CellTiter-Blue® Assay and other methods used to determine cell viability or cytotoxicity. Figure 7, Panel A, shows data generated using the CellTiter-Blue® Assay and depicts the effect of tamoxifen on HepG2 cells. Increasing concentrations of tamoxifen are toxic to HepG2 cells, resulting in a decrease in fluorescent signal. Figure 7, Panel A, also shows results obtained using the CellTiter 96® AQueous One Solution Assay, which uses the reduction of MTS tetrazolium to a colored formazan product to measure cell viability. The data demonstrate an excellent correlation between these two different methods for measuring reducing capacity of viable cells. For both assays, nonviable cells rapidly lose the ability to reduce the redox dye and do not generate a signal.

Figure 7, Panel B, shows a similar comparison between the CellTiter-Blue® Assay and the CellTiter-Glo® Luminescent Cell Viability Assay, which measures ATP. Again, the IC₅₀ values determined using both assays are similar. There is a good correlation between measuring the reducing capacity of cells and the total ATP content as markers of cell viability.

Figure 7, Panel C, shows a comparison of results obtained using the CellTiter-Blue® Assay and the CytoTox-ONE™ Homogeneous Membrane Integrity Assay, which measures release of lactate dehydrogenase (LDH)—a marker of membrane integrity commonly used as a cytotoxicity assay method.

There is an inverse correlation between resazurin reduction, a viability indicator, and release of LDH, a cytotoxicity indicator. The IC₅₀ values determined using all four of these assay methods are similar.

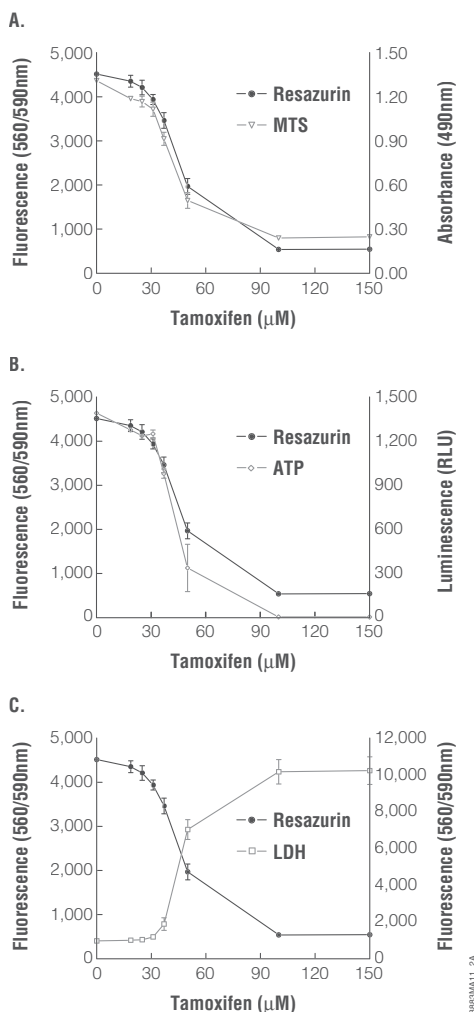


Figure 7. Comparison of the CellTiter-Blue® Assay with MTS-, ATP- and LDH-based methods for cytotoxicity testing. Results of cell viability assays using the CellTiter-Blue® Reagent were compared with those obtained using: **Panel A.** The MTS-based CellTiter 96® AQ_{ueous} One Solution Assay (Cat.# G3580); **Panel B.** The ATP-based CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570). **Panel C.** The LDH-based CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Cat.# G7890). All assays were performed according to the recommended protocols (10–12). HepG2 cells were seeded at 15,000 cells/well in 96-well plates in 90 μl MEM supplemented with 10% FBS, nonessential amino acids and 1mM sodium pyruvate, and were cultured for 24 hours at 37°C. Tamoxifen (0–150 μM) was diluted into culture medium. Plates designated for the CellTiter-Glo® (ATP) and the CytoTox-ONE™ (LDH) Assays were cooled to 22°C prior to adding reagents. Reagents for the CellTiter 96® (MTS) Assay and the CellTiter-Blue® Assay were added to the respective plates and incubated for 1 hour at 37°C.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

<u>Symptoms</u>	<u>Causes and Comments</u>
High background	Product has been exposed to light for extended periods. Do not expose the CellTiter-Blue® Reagent to direct light for extended amounts of time.
Fluorescence values above linear range	Too much reduction of resazurin. Repeat assay of plate reader and shorten the incubation time.
Low overall fluorescence	Low cell number or incubation time too short. Use more cells per well or increase incubation time. Plate reader set at incorrect gain. Adjust gain setting. Improper filter set. Choose appropriate excitation and emission filters. Verify their physical position in the reader and verify positioning in software control. Secondary reduction of resorufin to the nonfluorescent hydro-resorufin (see Section 3.A). Decrease incubation time with CellTiter-Blue® Reagent.

7. References

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7. References (continued)

8. Gonzalez, R.J. and Tarloff, J.B. (2001) Evaluation of hepatic subcellular fractions for Alamar Blue and MTT reductase activity. *Toxicology in Vitro* 15, 257–9.
9. *Apo-ONE® Homogeneous Caspase 3/7 Assay Technical Bulletin #TB295*, Promega Corporation.
10. *CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Technical Bulletin #TB245*, Promega Corporation.
11. *CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288*, Promega Corporation.
12. *CytoTox-ONE™ Homogeneous Cytotoxicity Assay Technical Bulletin #TB306*, Promega Corporation.

8. Related Products

PRODUCT	SIZE	CAT. #
CellTiter-Glo® Luminescent Cell Viability Assay (ATP)	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
	5 × 10ml	G9201
	2 × 50ml	G9202
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH)	200–800 assays	G7890
	1,000–4,000 assays	G7891
CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH)	1,000 assays	G1780
CellTiter 96® AQ _{ueous} Non-Radioactive Cell Proliferation Assay (MTS)	1,000 assays	G5421
	5,000 assays	G5430
	50,000 assays	G5440
CellTiter 96® AQ _{ueous} MTS Reagent Powder	1g	G1111
	250mg	G1112
CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT)	1,000 assays	G4000
	5,000 assays	G4100

9. Summary of Changes

The following change was made to the 3/16 revision of this Technical Bulletin, TB317:

The x axis label on Figure 3 was revised to “× 10³”.

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